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<p><b>(54) Title:</b> CATALYTIC NUCLEIC ACID-BASED DIAGNOSTIC METHODS</p> <p><b>(57) Abstract</b></p> <p>This invention provides methods and kits useful for determining whether a subject is afflicted with a disorder characterized by the presence of one or more known nucleic acid mutations. The instant methods comprise steps of nucleic acid molecule isolation, amplification, contact with one or more catalytic nucleic acid molecules specifically cleaving a target sequence present either in the case of disorder or wild-type, but not both, and determining cleavage of the amplified segment(s).</p>			

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CATALYTIC NUCLEIC ACID-BASED DIAGNOSTIC METHODS

5       Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

10

Field of the Invention

This invention relates to methods of diagnosing disorders characterized by known nucleic acid mutations.

15      The instant methods employ the use of catalytic nucleic acid molecules, and are useful in connection with diagnosing such disorders as cancer and AIDS.

Background of the Invention

20

A variety of inherited and acquired diseases are associated with genetic variations such as point mutations, deletions and insertions. Some of these variations are directly associated with the presence of disease, while others correlate with disease risk and/or prognosis. There are more than 500 human genetic diseases which result from mutations in single genes (21, 22). These include cystic fibrosis, muscular dystrophy,  $\alpha 1$ -antitrypsin deficiency, phenylketonuria, sickle cell anemia or trait, and various other hemoglobinopathies (21, 22). Furthermore, individuals with increased susceptibility to several common polygenic conditions, such as atherosclerotic heart disease, have been shown to have an association with the inheritance of particular DNA sequence polymorphisms.

Cancer is thought to develop due to the accumulation of genetic lesions in genes involved in cellular proliferation or differentiation. The *ras* proto-oncogenes, *K-ras*, *N-ras* and *H-ras*, and the p53 tumor suppressor gene are examples of genes which are frequently mutated in human cancers. Specific mutations in these genes leads to an increase in transforming potential. Genetic analysis would be invaluable in the clinic for assessing disease risk, diagnosis of disease, predicting a patient's prognosis or response to therapy, and monitoring a patient's progress. The introduction of such genetic tests, however, will depend on the development of simple, inexpensive, and rapid assays for genetic variations.

15

Methods of *in vitro* nucleic acid amplification have wide-spread applications in genetics and disease diagnosis. In the last decade many techniques for amplification of nucleic acid have been described. These include the polymerase chain reaction (PCR) (1-7), the ligase chain reaction (LCR) (8), the strand displacement amplification assay (SDA) (9) and transcription-mediated amplification (TMA) (10, 11) (also known as self-sustained sequence replication (SSR)). The amplification products (amplicons) produced by PCR, LCR and SDA are DNA, whereas RNA amplicons are produced by TMA. DNA or RNA templates, generated by these protocols or others, can be analyzed for the presence of sequence variation (i.e. mutation) associated with the disease to be ascertained.

As with nucleic acid amplification, catalytic nucleic acids have been studied intensively in recent years. The potential for suppression of gene function using catalytic nucleic acids as therapeutic agents is widely discussed in the literature (12-18). Catalytic

RNA molecules (ribozymes) have been shown to be capable of cleaving both RNA (12) and DNA (17) molecules. Similarly, catalytic DNA molecules (DNAzymes) have also been shown to be capable of cleaving both RNA (13, 19) 5 and DNA (18) molecules. Catalytic nucleic acid can only cleave a target nucleic acid sequence, provided that target sequence meets minimum sequence requirements. The target sequence must be complementary to the hybridizing regions of the catalytic nucleic acid and 10 the target must contain a specific sequence at the site of cleavage. Examples of such sequence requirements at the cleavage site include the requirement for a purine:pyrimidine sequence for a class of DNAzyme 15 cleavage (10-23 model) (19), and the requirement for the sequence uridine:H where H can equal A, C or U but not G, for the hammerhead ribozymes (23).

In addition to their therapeutic potential, catalytic nucleic acid molecules can also distinguish 20 between targets which differ by a single point mutation (14-16). This is achieved by targeting a specific sequence which is present in wild-type but not mutant templates or vice versa. So far, this capacity for discrimination has only been exploited as a method for 25 therapeutic manipulation of gene expression.

A review by Nollau-Wagener (24) compared several methodologies for the detection of point mutations with respect to the type of nucleic acid analyzed, the 30 percentage of mutations detected, the time and cost of performing the assay, and problems relating to the use of toxic reagents. Each of the methodologies examined had its drawbacks. For example, denaturing gradient gel electrophoresis is time consuming, RNAase A cleavage can 35 only detect about 70% of possible mutations, and chemical cleavage involves the use of toxic substances.

Another method, known as restriction fragment length polymorphism (RFLP), involves ascertaining whether a restriction enzyme site is present or absent 5 at the locus of interest. In rare instances, mutations can be detected because they happen to lie within a naturally occurring restriction endonuclease recognition/cleavage site (31).

10 The inclusion of mismatched bases within primers used to facilitate *in vitro* amplification can result in the induction of artificial restriction endonuclease recognition/cleavage sites, and hence an increase in the number of loci which can be analyzed by RFLP (32).  
15 Modified primers containing mismatched bases have been used to induce artificial recognition/cleavage sites for restriction endonucleases at critical codons within the *ras* gene family (33-35). The general rules for designing primers which contain mismatched bases located  
20 near the 3' termini of primers have been established (36).

Although the use of mismatched primers has expanded the utility of RFLP analysis, the technique is still limited by the fact that a minimum of four base pairs is required for recognition and cleavage by a restriction 25 enzyme.

Summary of the Invention

This invention provides a method of determining whether a subject is afflicted with a disorder characterized by the presence of a known nucleic acid mutation, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the mutation in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining whether the catalytic nucleic acid molecule in step (b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

This invention also provides a method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding

wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in  
5 step (b)(ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

This invention further provides a method of  
10 determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segments present in the isolated sample, which segments collectively are known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segments with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in one of the nucleic acid segments having one of the known mutations or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be  
15 performed either subsequent to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in step (b)(ii) cleaves the amplified segment containing its respective target sequence, so as to determine whether the subject is afflicted with the disorder.  
20  
25  
30

Finally, this invention provides kits for use in practicing the instant diagnostic methods. The first instant kit comprises (a) a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid  
35

segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a nucleic acid reagent suitable for use in amplifying the nucleic acid 5 segment containing the target sequence.

The second instant kit comprises (a) a 10-23 DNAzyme which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid 10 segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a DNA primer suitable for initiating amplification of the segment under polymerase chain reaction conditions, which primer 15 contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme.

20 The third instant kit comprises (a) a first DNA primer which comprises a zymogene encoding a 10-23 DNAzyme that specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of 25 a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, which first primer is suitable for initiating amplification of the segment under polymerase chain reaction conditions; and (b) a second DNA primer suitable for initiating amplification 30 of the segment under polymerase chain reaction conditions, which second primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme, such that, upon

amplification, (i) the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme, and (ii) the amplified nucleic acid segment is recognized and cleaved *in cis* by the DNAzyme.

Detailed Description of the Invention

This invention provides methods employing catalytic nucleic acids to determine whether a subject is afflicted 5 with a disorder characterized by the presence of one or more known nucleic acid mutations. These methods are collectively applicable to scenarios where the disorder is characterized by (i) a single mutation within a single nucleic acid segment, or (ii) a plurality of mutations 10 within a single nucleic acid segment, or (iii) a plurality of mutations within a plurality of nucleic acid segments. For each mutation tested for by nucleic acid amplification, specific cleavage, and analysis, the instant methods provide a "yes or no" answer as to whether 15 the mutation exists. This answer in turn ultimately leads to a "yes or no" answer as to whether the corresponding disorder is present in the subject.

Specifically, this invention provides a method of 20 determining whether a subject is afflicted with a disorder characterized by the presence of a known nucleic acid mutation, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment present in the 25 isolated sample, which segment is known to contain the mutation in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a catalytic nucleic acid molecule which specifically recognizes and cleaves a target 30 sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining 35 whether the catalytic nucleic acid molecule in step

(b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

This invention also provides a method of determining  
5 whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment  
10 present in the isolated sample, which segment is known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a plurality of catalytic nucleic acid molecules, each of  
15 which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent  
20 to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in step (b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

25

This invention further provides a method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a)  
30 isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segments present in the isolated sample, which segments collectively are known to contain the plurality of mutations in a subject afflicted with the disorder, and  
35 (ii) under suitable conditions, contacting the resulting amplified segments with a plurality of catalytic nucleic

acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in one of the nucleic acid segments having one of the known mutations or (2) in the corresponding wild-type nucleic acid segment,

5 but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in step (b)(ii) cleaves the amplified segment containing its respective target

10 sequence, so as to determine whether the subject is afflicted with the disorder.

The instant methods can be used to diagnose disorders in any subject. As used herein, "subject" means any

15 animal, including, for example, mice, rats, dogs, guinea pigs, ferrets, rabbits, and primates. In the preferred embodiment, the subject is a human.

The disorder diagnosed by the instant invention can

20 be any disorder characterized by the presence of at least one known nucleic acid mutation, which mutation is missing when such disorder is absent. Such disorders are well known in the art and include, by way of example, cancer, AIDS, cystic fibrosis, muscular dystrophy,  $\alpha$ l-anti-

25 trypsin deficiency, phenylketonuria, sickle cell anemia or trait, and various other hemoglobinopathies. In one embodiment, the disorder is selected from the group consisting of cancer, AIDS, and cystic fibrosis. In the preferred embodiment, the disorder is cancer. In the

30 Experimental Details section which follows, numerous examples are given of specific mutations, target sequences containing same, and catalytic nucleic acids used for diagnosing such disorders as cancer, AIDS and cystic fibrosis.

As used herein, "catalytic nucleic acid molecule" means a DNA molecule (also known in the art as a "DNAzyme") or RNA molecule (also known in the art as a "ribozyme") which specifically recognizes and cleaves a 5 distinct target nucleic acid sequence. For both DNAzymes and ribozymes, the target nucleic acid sequence can be either DNA or RNA.

The nucleic acid sequence in which the known 10 disorder-characterizing mutation(s) resides (i.e., the sequence amplified in the instant methods) can be a DNA or RNA sequence. These mutation(s) include, for example, point mutations, deletion mutations, insertion mutations and frame-shift mutations. Each of the amplified nucleic 15 acid segment and catalytic nucleic acid molecule can be either DNA or RNA. In one embodiment, the amplified nucleic acid segment is RNA and the catalytic nucleic acid molecule is either DNA or RNA. In a further embodiment, the amplified nucleic acid segment is DNA and the 20 catalytic nucleic acid molecule is either RNA or DNA (25).

Methods for isolating and amplifying nucleic acid molecules used in the instant invention are well known in the art. More specifically, methods of isolating a sample 25 of nucleic acid molecules from the subject include, for example, phenol chloroform extraction, quick lysis, capture on columns and polymer capture (20, 26-29). Methods of amplifying a nucleic acid sequence include, for example, PCR, LCR, SDA and TMA (also known as (SSR)) (1- 30 11).

Suitable conditions for contacting an amplified nucleic acid segment containing a target sequence with a catalytic nucleic acid molecule so as to permit specific 35 recognition and cleavage of the target sequence are well

known in the art. In addition, such conditions are exemplified in the Experimental Details section below.

Methods of determining whether a catalytic nucleic acid molecule cleaves an amplified nucleic acid segment are also routine in the art. Such methods include, by way of example, polyacrylamide gel electrophoresis and capillary electrophoresis (20, 30).

In the preferred embodiment of this invention, (a) the amplification is performed using a polymerase chain reaction; (b) the catalytic nucleic acid molecule is a 10-23 DNAzyme; and (c) the polymerase chain reaction employs a DNA primer (i.e., a "chimeric" primer) suitable for initiating amplification of the segment, which primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme. This purine ribonucleotide residue in the chimeric primer is required for cleavage by the 10-23 DNAzyme. Thus, using this chimeric primer permits the 10-23 DNAzyme cleavage site to be generated in a PCR reaction. The chimeric primer can also include, for example, a ribonucleotide residue that serves as the 3' side of the site recognized and cleaved by the 10-23 DNAzyme.

In one form of this embodiment, the amplified segment is recognized and cleaved in *trans* by the DNAzyme. In another form, (a) the polymerase chain reaction employs a second DNA primer suitable for initiating amplification of the segment, which second primer comprises a zymogene encoding a 10-23 DNAzyme such that, upon amplification, the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme; and (b) the amplified nucleic acid segment is recognized and cleaved in *cis* by the DNAzyme.

As used herein, "cis" cleavage by a DNAzyme shall mean that the DNAzyme recognizes and cleaves a sequence coexisting therewith on the same amplified nucleic acid molecule. Trans cleavage shall mean that the DNAzyme cleaves a substrate located on a different molecule. Finally, "zymogene" shall mean a nucleic acid sequence which comprises the anti-sense (i.e. complementary) sequence of a catalytic nucleic acid molecule, and whose transcription product is the catalytic nucleic acid molecule itself.

This invention still further provides kits for use in practicing the instant diagnostic methods. The first instant kit comprises (a) a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a nucleic acid reagent suitable for use in amplifying the nucleic acid segment containing the target sequence.

In one embodiment, the kit comprises a plurality of catalytic nucleic acid molecules. The nucleic acid reagent suitable for use in amplifying the nucleic acid segment containing the target sequence can be, for example, a nucleic acid primer. In one embodiment, the kit comprises a plurality of such nucleic acid reagents.

More specifically, the second instant kit comprises (a) a 10-23 DNAzyme which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a DNA primer

suitable for initiating amplification of the segment under polymerase chain reaction conditions, which primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the 5 amplified segment recognized and cleaved by the 10-23 DNAzyme.

The third instant kit comprises (a) a first DNA primer which comprises a zymogene encoding a 10-23 10 DNAzyme that specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, which first primer 15 is suitable for initiating amplification of the segment under polymerase chain reaction conditions; and (b) a second DNA primer suitable for initiating amplification of the segment under polymerase chain reaction conditions, which second primer contains at least one 20 purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme, such that, upon amplification, (i) the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme, and (ii) the 25 amplified nucleic acid segment is recognized and cleaved *in cis* by the DNAzyme.

In one embodiment, the instant kits further comprise one or more of the following: (a) reagents 30 useful for isolating a sample of nucleic acid molecules from a subject being diagnosed; (b) reagents useful for amplifying a nucleic acid segment present in the isolated sample, which segment is known to contain a mutation in a subject afflicted with the disorder; and (c) reagents 35 useful for creating suitable reaction conditions for catalytic nucleic acid activity. The reagents in

components (a)-(c) of the instant kits can either be obtained commercially or made according to well known methods in the art, as exemplified in the Experimental Details section below.

5

The components of the instant kits can be in solution or lyophilized as appropriate. In one embodiment, the components of the instant kits are in the same compartment, and in another embodiment, the 10 components of the instant kit are in separate compartments. In the preferred embodiment, the kits further comprise instructions for use.

This invention will be better understood by reference 15 to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

The examples of DNAzymes and ribozymes listed below  
 5 are based on a 10-23 DNAzyme (19) and are designed to  
 cleave the following medically important targets. The  
 examples of ribozymes listed below are based on the  
 hammerhead ribozyme (12).

10 I. DNAzymes

where R = purine, either A or G.  
 Y = pyrimidine, either C, T or U.  
 H = A, T, U, or C (not G).  
 15 D = A, T, U, or G (not C).  
 B = C, T, U, or G (not A).  
 V = A, C, or G (not T, not U).  
 W = T, U, or A.  
 italics = bases which are artificially  
 20 introduced by in vitro amplification  
 using primers containing bases which are  
 mismatched with respect to the target  
 sequence.  
 bold = target base or sequence for  
 25 analysis.  
 underlined = recognition site (RY/R).  
 E = modified sequence (primer-induced  
 artificial sequence).

30 A. Acquired Disease(1) Cancer(a) K-ras codon 12, position 2 - mutant (G to C, U or A)

35           5' - GUU GGA GCU GGU GGC GUA GGC - 3'      wildtype  
                 RNA  
          5' - GUU GGA GCU GYU GGC GUA GGC - 3'      mutant RNA  
          3' - CAA CCT CGA RA CCG CAT CCG - 5'      DNAzyme  
 40            A    G  
               G    G  
               C    C  
               A    T  
               A    A  
 45            C    G  
               A    C  
               T

5' - GUU GGA GCU GAU GGC GUA GGC - 3'      mutant RNA  
          3' - CAA CCU CGA CUA CCG CAU CCG - 5'      antisense

5      5' - GCC UAC GCC AUC AGC UCC AAC - 3'      antisense  
          3' - CGG ATG CGG AG TCG AGG TTG - 5'      DNAzyme

10      A    G  
               G    G  
               C    C  
               A    T  
               A    A  
               C    G  
               A    C  
               T

15      (b) K-ras codon 13, position 1 - mutant (G to A, U or C)

20      5' - GGA GCU GGU GGC GUA GGC AAG - 3'      wildtype RNA  
           5' - GGA GCU GGU HGC GUA GGC AAG - 3'      mutant RNA  
           3' - CCT CGA C A DCG CAT CCG TTC - 5'      DNAzyme

25      A    G  
               G    G  
               C    C  
               A    T  
               A    A  
               C    G  
               A    C  
               T

30      (c) H-ras codon 61, position 1 - mutant (C to G, U or A)

35      5' - ACC GCC GGC CAG GAG GAG - 3'      wildtype RNA  
           5' - ACC GCC GGC DAG GAG GAG - 3'      mutant RNA  
           3' - TGG CGG C G HTC CTC CTC - 5'      DNAzyme

40      A    G  
               G    G  
               C    C  
               A    T  
               A    A  
               C    G  
               A    C  
               T

45      (d) H-ras codon 61, position 1 - mutant (C to G, U or A)

(d) H-ras codon 61, position 2 - mutant (A to C, G or U)

	5' - ACC GCC GGC CAG GAG GAG - 3'	wildtype RNA
5	3' - UGG CGG CCG <u>GUC</u> <u>GUC</u> CUC - 5'	<u>E</u> wildtype RNA
	5' - CUC <u>CUG</u> <u>CUG</u> GCC GGC GGU - 3'	<u>E</u> wildtype RNA
10	3' - GAG GA GAC CGG CCG CCA - 5'	DNAzyme
	A      G G      G C      C A      T 15      A      A C      G A      C T	

20 (e) H-ras codon 61, position 3 - mutant (G to C or U)

(Note; G to A is a silent mutation)

	5' - ACC GCC GGC CAG GAG GAG - 3'	wildtype RNA
25	3' - UGG CGG CCG <u>GUR</u> CUC CUC - 5'	mutant RNA antisense
	5' - CUC CUC <u>RUG</u> GCC GGC GGU - 3'	antisense
30	3' - GAG GAG <u>AC</u> CGG CCG CCA - 5'	DNAzyme
	A      G G      G C      C A      T 35      A      A C      G A      C T	

40 (f) N-ras codon 61, position 1 - mutant (C to A, G or U)

	5' - GCU GGA CAA GAA GAG - 3'	wildtype RNA
45	3' - CGA CCU HUG CUU CUC - 5'	mutant RNA <u>E</u> mutant RNA

E mutant RNA  
DNAzyme

5' - CUC UUC <u>GUH</u> UCC AGC - 3' 3' - GAG AAG <u>AD</u> AGG TCG - 5' A    G 5              G    G C            C A            T A            A C            G 10             A    C T	G    G C    C A    T A    A C    G 15             A    C T
--	--

mutant RNA  
DNAzyme

5' - GCU GGA UAA GAA GAG - 3' 3' - CGA CC <u>ATT</u> CTT CTC - 5' A    G 15              G    G C            C A            T A            A 20             C    G A    C T	G    G C    C A    T A    A 25             C    G A    C T
--	--

(2) HIV 1 - AZT Resistance, Point Mutations

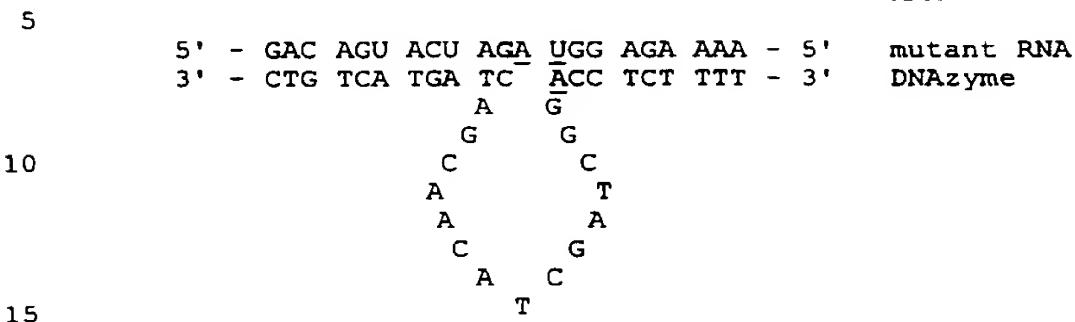
wildtype  
RNA

mutant RNA  
DNAzyme

5' - UGU ACA GAA AUG GAA AAG - 3' A    G 30              G    G C            C A            T A            A C            G 35             A    C T	G    G C    C A    T A    A C    G 40             A    C
---	---

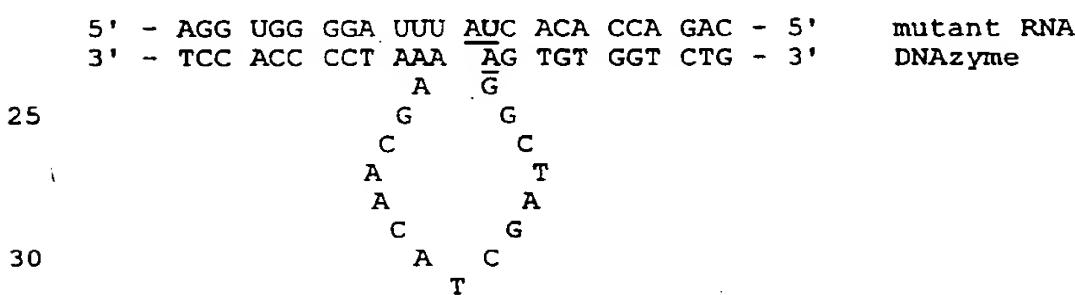
(b) Codon 70 - mutant (A to G)

5' - GAC AGU ACU AAA UGG AGA AAA - 3' wildtype  
RNA

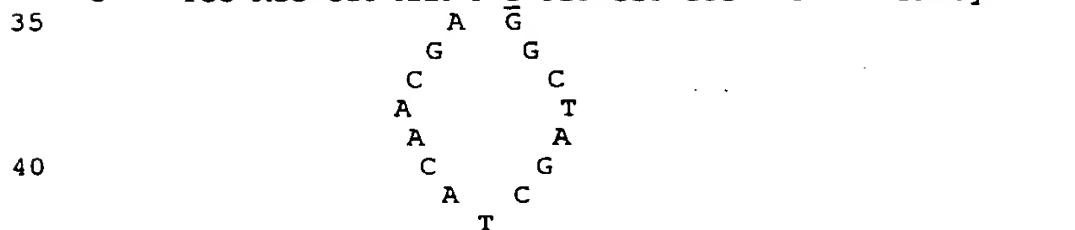


(c) Codon 215 - mutant (C to U or A)

5' - AGG UGG GGA UUU ACC ACA CCA GAC - 3' wildtype RNA

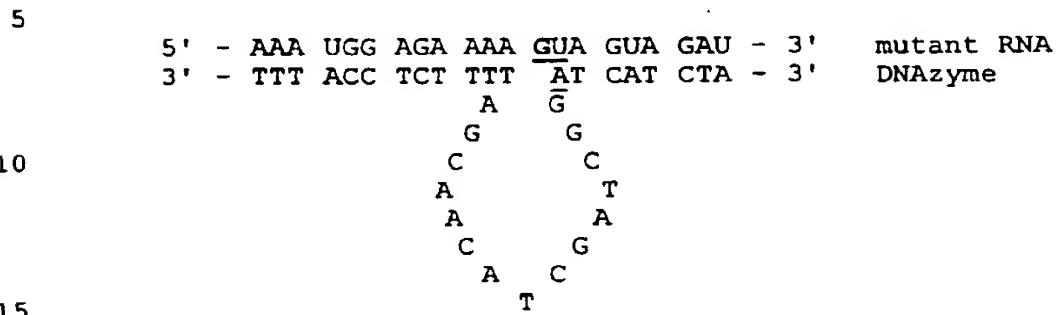


5' - AGG UGG GGA UUU AAC ACA CCA GAC - 5'      mutant RNA  
 3' - TCC ACC CCT AAA T G TGT GGT CTG - 3'      DNAzyme

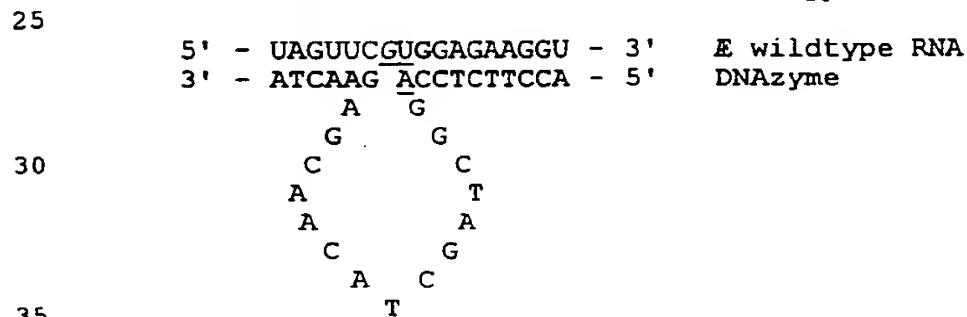


(d) Codon 74 - mutant (U to G confers ddT resistance)

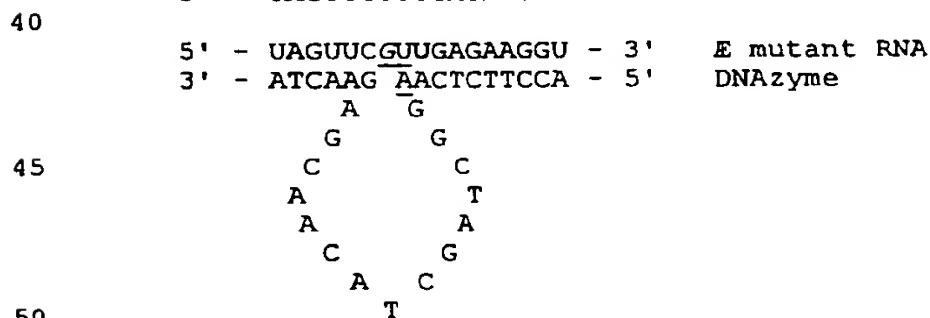
5' - AAA UGG AGA AAA UUA GUA GAU - 3' wildtype RNA

B. Inherited Disease20 (1) Cystic Fibrosis(a) Codon 542 - wildtype

5' - UAGUUUCUUGGAGAAGGU - 3' wildtype RNA

Codon 542 - mutant (G to U)

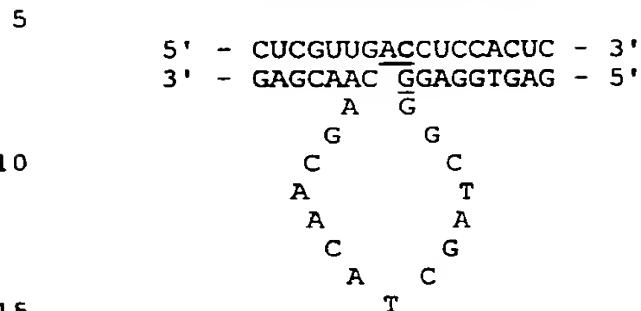
40 5' - UAGUUUCUUUGAGAAGGU - 3' mutant RNA



(b) Codon 551 - wildtype

5' - GAGUGGAGGUCAACGAG - 3'  
 3' - CUCACCUCAGUUGCUC - 5'

wildtype RNA  
 antisense

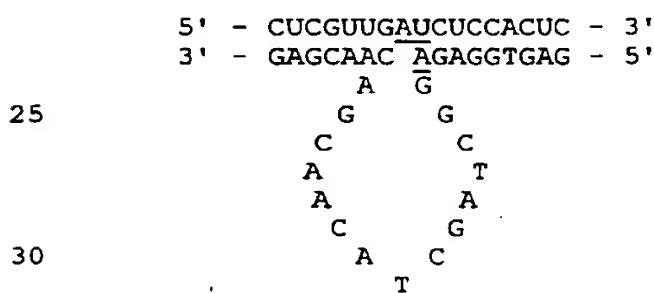


antisense  
 DNAzyme

Codon 551 - mutant (G to A)

5' - GAGUGGAGAUCAACGAG - 3'  
 3' - CUCACCUCUAGUUGCUC - 5'

mutant RNA  
 antisense

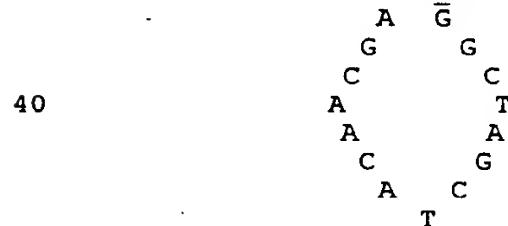


antisense  
 DNAzyme

(c) Codon 508 - wildtype

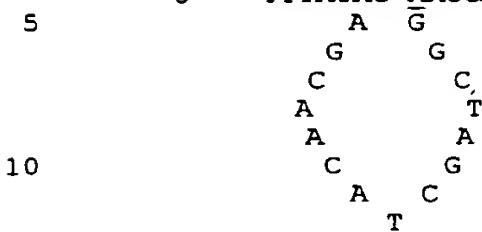
35 5' - GAAUAUCAUCUUUGGUGUUU - 3'  
 3' - CTTTATAG AGAAACCACAAA - 5'

wildtype RNA  
 DNAzyme



Codon 508 - mutant (CTT deletion)

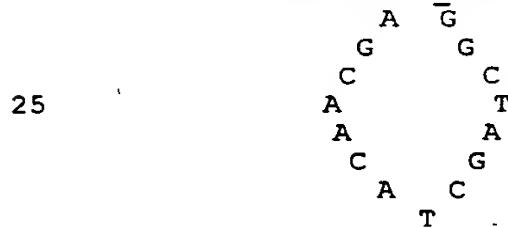
5' - AAAUAUCAUUGGUGUUU - 5'                  mutant RNA  
 3' - TTTATAG AACCACAAA - 3'                  DNAzyme

(2) α<sub>1</sub>-antitrypsin

15                  Codon 342 - mutant (G to A)

5' - GACCAUCGACGAGAAAGG - 3'                  wildtype RNA

20                  5' - GACCAUCGACAAGAAAGG - 3'  
 3' - CTGGTAGC GTTCTTC - 5'                  mutant RNA  
 DNAzyme



II. Ribozymes

5 where bold = target base for analysis.  
underlined = recognition site (UH).

A. Acquired Disease(1) Cancer10 K-ras codon 12, position 1 - mutant (G to A, C or U)

15           5' - GUA GUU GGA GCU GGU GGC GUA - 3'      wildtype  
RNA  
5' - GUA GUU GGA GCU HGU GGC GUA - 3'      mutant RNA  
3' - CAU CAA CCU CGA CA CCG CAU - 5'      Ribozyme  
A   C  
A   U  
20           G   A   G  
C   G   G   A  
A   U   U  
G   C  
G   C  
25           A   G  
G   U

K-ras codon 12, position 2 - mutant (G to U)

30           5' - GUU GGA GCU GGU GGC GUA GGC- 3'      wildtype  
RNA  
5' - GUU GGA GCU GUU GGC GUA GGC- 3'      mutant RNA  
3' - CAA CCU CGA CA CCG CAU CCG- 5'      Ribozyme  
35           A   C  
A   U  
G   A   G  
C   G   G   A  
A   U   U  
40           G   C  
G   C  
A   G  
G   U

45 (2) HIV 1 - AZT resistance(a) Codon 41 - mutant (A to U or C)

50           5' - UGU ACA GAA AUG GAA AAG - 3'      wildtype  
RNA  
5' - UGU ACA GAA YUG GAA AAG - 3'      mutant RNA  
3' - ACA UGU CUU RAC CUU UUC - 5'      antisense

antisense  
Ribozyme

	5'	- CUU UUC CAR <u>UUC</u> UGU ACA - 3'	
	3'	- GAA AAG GUY A G ACA UGU - 5'	
		A C	
		A U	
5		G A G	
		C G G A	
		A U U	
		G C	
		G C	
10		A G	
		G U	

(b) Codon 70 - mutant (A to G)

15	5'	- GAC AGU ACU AAA UGG AGA AAA - 3'	wildtype RNA
	3'	- CUG UCA UGA UCU ACC UCU UUU - 5'	
20	5'	- UUU UCU CCA <u>UCU</u> AGU ACU GUC - 3'	mutant RNA
	3'	- AAA AGA GGU AGA CA UGA CAG - 5'	antisense
		A C	
		A U	
25		G A G	
		C G G A	
		A U U	
		G C	
		G C	
30		A G	
		G U	

(c) Codon 215 - mutant (C to U or A)

35	5'	- AGG UGG GGA UUU ACC ACA CCA GAC - 3'	wildtype RNA
	3'	- UCC ACC CCU AAA UWG UGU GGU CUG - 5'	
40	5'	- AGG UGG GGA UUU AWC ACA CCA GAC - 3'	mutant RNA
	3'	- UCC ACC CCU AAA UWG UGU GGU CUG - 5'	antisense
		A C	
		A U	
45		G A G	
		C G G A	
		A U U	
		G C	
		G C	
50		A G	
		G U	

(d) Codon 74 - mutant (U to G confers ddT resistance)

5' - AAA UGG AGA AAA UUA GUA GAU - 3'      wildtype  
RNA

5  
5' - AAA UGG AGA AAA GUA GUA GAU - 3'      mutant RNA  
3' - UUU ACC UCU UUU CA CAU CUA - 5'      Ribozyme

10  
A      C  
A      U  
G      A      G  
C      G      G      A  
A      U      U  
G      C  
G      C  
15  
A      G  
G      U

B. Inherited Disease20 (1) Cystic Fibrosis(a) Codon 542 - wildtype

25 5' - UAGUUCUUGGAGAAGGUGGA - 3'      wildtype  
RNA

3' - AUCAAGA CCUCUUCCACCU - 5'      Ribozyme

30  
A      C  
A      U  
G      A      G  
C      G      G      A  
A      U      U  
G      C  
G      C  
35  
A      G  
G      U

Codon 542 - mutant (G to U)

40 5' - UAGUUCUUUGAGAAGGU - 5'      mutant RNA

3' - AUCAAGA ACUCUUCCA - 3'      Ribozyme

45  
A      C  
A      U  
G      A      G  
C      G      G      A  
A      U      U  
G      C  
G      C  
A      G  
G      U

(b) Codon 551 - wildtype

	5'	- GAGUGGAGGU <u>C</u> AACGAG - 3'	wildtype RNA Ribozyme
5	3'	- CUCACCUC <u>CA</u> UUGCUC - 5'	
		A   C	
10		A   U	
		G A   G	
		C G G A	
		A U   U	
		G C	
15		G C	
		A   G	
		G U	
		<u>Codon 551 - mutant (G to A)</u>	
	5'	- GAGUGGAG <u>A</u> UCAACGAG - 3'	mutant RNA
20	3'	- CUCACC <u>C</u> UCA UUGCUC - 5'	Ribozyme
		A   C	
		A   U	
25		G A   G	
		C G G A	
		A U   U	
		G C	
		G C	
		A   G	
30		G U	
	(c)	<u>Codon 508 - wildtype</u>	
	5'	- GAAAUA <u>C</u> U <u>C</u> UUGGUGUUU - 3'	wildtype RNA
35	3'	- CUUUAU <u>G</u> U <u>A</u> GA ACCACAAA - 5'	Ribozyme
		A   C	
		A   U	
40		G A   G	
		C G G A	
		A U   U	
		G C	
		G C	
45		A   G	
		G U	
		or	

### Codon 508 - mutant (CUU deletion)

5' - GAAAUAUCAUUGGUGUUU - 3'                          mutant RNA  
 3' - CUUUAUAGUA CCACAAA - 5'                          Ribozyme

S	A C
	A U
	G A G
	C G G A
10	A U U
	G C
	G C
	A G
	G U

## mutant RNA Ribozyme

15 (2)  $\beta$ -Globin

B+ - black (poly A signal) - mutant (U to C)

5' - UCUGCCUAUUAAAAACAU - 3' wildtype  
20 RNA

5' - UCUGCCUAACAAAAACAU - 3'                          mutant RNA  
 3' - AGACGGAUUGUUUUUUGUA - 5'                          antisense

30                    A C  
                      A U  
                      G A G  
                      C G G A  
                      A U U  
                      G C  
                      G C  
                      A G  
                      G U  
35

### III. K-ras Analysis Using Ribozymes

40 A. Ribozymes Targeting Mutations at K-ras Codon 12

The sequence of the human K-ras gene at codon 12 is GGT. Point mutations are frequently observed in the first 2 bases in this sequence in association with cancer of the pancreas, lung and colon. Two ribozymes were designed to cleave mutant but not wild-type K-ras.

K-ras                    5'••••AGUUGGAGCUHGUGGC GUAGG•••• 3'  
 Ribozyme I            3'        UCAACCUCGA CACCGCAUCC            5'  
 .                      A     C  
 5                      A     U  
 .                      G     A     G  
 .                      C     G     G     A  
 .                      A     U     U  
 .                      G     C  
 10                     G     C  
 .                      A     G  
 .                      G     U

(K-ras codon 12 - bold; Ribozyme target doublet -  
 15 underlined)

Ribozyme I, above, is designed to cleave all RNA molecules which contain a point mutation at the first position of codon 12, but is designed not to cleave the wild-type sequence. The target sequence for the ribozyme is UH where H can equal C, U or A, but not G. Since the wild-type sequence is G at this position, all mutations will be cleaved with this ribozyme.

25                      K-ras                    5'••••UUGGAGCUGUGGCGUAGGCA•••• 3'  
 Ribozyme II            3'        AACCUCGACA CCGCAUCCGU            5'  
 .                      A     C  
 .                      A     U  
 30                     G     A     G  
 .                      C     G     G     A  
 .                      A     U     U  
 .                      G     C  
 .                      G     C  
 35                     A     G  
 .                      G     U

(K-ras codon 12 mutant allele - bold; Ribozyme target doublet - underlined)

Ribozyme II is designed to target G to U substitutions at position 2 of codon 12. The wild-type sequence G cannot base pair with the A at the first 45 position within the hybridizing arms of the ribozyme,

and hence the wild-type sequence is not expected to be cleaved with this ribozyme.

DNA sequences encoding ribozymes I and II were  
5 synthesized by Macromolecular Resources (Fort Collins,  
CO). The antisense and sense strands of the ribozymes  
were annealed and cloned into the vector pSP70 (Promega  
Corporation, Madison, WI) behind the T7 polymerase  
promoter. These clones were linearized at a site 3' to  
10 the ribozyme by digestion with *Nde* I, then purified.  
Radiolabelled ribozymes were prepared by standard *in*  
*vitro* RNA transcription reactions which incorporated  
[alpha-<sup>32</sup>P] UTP using these templates (20).

15    B.    Preparation of K-ras Templates

The human cell lines SW480 and Calu 1 were obtained  
from the American Type Culture Collection (Rockville,  
MD). The colon carcinoma cell line SW480 has a  
20 homozygous mutation (GTT) at position 2 within codon 12  
of K-ras. Calu 1 is a lung carcinoma cell line which is  
heterozygous at position 1 within K-ras codon 12 having  
both wild-type (GGT) and mutant (TGT) alleles.

25    K-ras DNA templates (4 mutant and 1 wild-type at  
codon 12) were generated by PCR amplification of Calu 1  
and SW480 DNA, as well as pUC plasmid clones which  
contained K-ras inserts that were mutant at codon 12.  
The sequence of the 5' PCR primer was TGGACTTAATACGA  
30    CTCACTATAGGGCGACTGAATATAAACTTGTGGTAG. This 5' primer  
incorporated the T7 promoter at the 5' end. The  
sequence of the 3' primer was CCTCTATTGTTGGATCATATTG.  
Radiolabelled K-ras RNA templates were generated by  
using the T7/K-ras PCR products in standard *in vitro* RNA  
35    transcription reactions which incorporated [alpha-<sup>32</sup>P]  
UTP (20).

C. Detection of Point Mutations

In vitro cleavage experiments were performed as follows. Ribozyme and substrate were incubated in cleavage buffer (10mM MgCl<sub>2</sub>; 250mM Tris.Cl, pH 7.5) in a 4:1 molar ratio. The ribozymes I and II were incubated with the radiolabelled K-ras RNA templates at 50°C for 6hr to assess *in vitro* cleavage ability. Reactions were analyzed by polyacryamide gel electrophoresis. Ribozyme I successfully cleaved K-ras RNA which contained a C, A or U mutation at codon 12 position 1 but was unable to cleave the wild-type sequence G. Ribozyme II successfully cleaved K-ras RNA which contained a U mutation at codon 12 position 2 but was unable to cleave the wild-type sequence G. The presence of cleaved K-ras RNA is therefore diagnostic for the presence of point mutations at codon 12.

This analysis demonstrates the ability of catalytic nucleic acids to specifically cleave templates *in vitro* which provides the basis for diagnose the presence of mutant sequences associated with disease. Nucleic acid can be amplified by a variety of techniques, e.g., PCR or TMA, and then cleaved with catalytic nucleic acids, e.g., DNAzyme (10-23 model), or ribozyme. The method can be used for detection of point mutations in K-ras which are specifically associated with cancer of the lung, colon and pancreas. The approach can be applied to diagnosis of any disease which is characterized by the presence of either an acquired or inherited genetic mutation.

IV. K-ras Mutation Analysis Using 10-23 DNAzymes and ChimERIC PrimERS

Walder, et al. (38) have previously shown that *Taq* 5 DNA polymerase can extend DNA/RNA chimeric primers that contain one or two 3' terminal ribose residues. Santoro and Joyce (19) showed cleavage of DNA/RNA chimeric substrates by the 10-23 DNAzyme. ChimERIC primERS are used here to produce PCR amplicons that serve as 10 substrates for the 10-23 DNAzyme.

A. Use of DNAzymes for distinguishing variant alleles; targeting sequences with DNAzymes supplied in trans

15 Cleavage of a DNAzyme substrate produced from a chimeric primer can be achieved by adding a chemically synthesised DNAzyme to the PCR mix. In such a reaction, the DNAzyme cleaves the substrate in the *trans* 20 orientation.

(1) DNAzymes targeting mutations at K-ras codon 12; natural cleavage site

25 (a) Strategy

PCR using a 5' DNA/RNA chimeric primer (5K42r) and a 3' primer (3K2) amplified a region of the K-ras gene. 30 5K42r hybridized to the K-ras sequence adjacent to codon 12 and contained the purine:pyrimidine residues which formed the potential DNAzyme cleavage site. The chimeric primer is fully complementary to the K-ras sequence that thus provided a natural cleavage site for a 10-23 DNAzyme. Extension from the 3' end of 5K42r by *Taq* DNA 35 polymerase amplified codon 12 of the K-ras gene. A DNAzyme, Dz1, was designed to cleave amplicons that harbor wild-type sequence at codon 12 of K-ras. The 5' arm of the DNAzyme was fully complementary to sequences that are wild-type at codon 12. Mutations at K-ras

codon 12, which result in mismatches with the 5' DNAzyme-hybridizing arm, were predicted to significantly decrease the efficiency of DNAzyme cleavage.

5 (b) Primer and DNAzyme Sequences

5' TATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGC<sub>AA</sub> 3' K-ras  
 5' TATAAACTTGTGGTAGTTGGAgcT 3' "5K42r" Primer  
 10 3' TGAACACCATCAACCT GACCACCG 5' "Dz1" DNAzyme  
 A G  
 G G  
 C C  
 15 A T  
 A A  
 C G  
 A T C

(Codon 12 in the K-ras wild-type sequence is underlined  
and the ribonucleotide bases in the primer 5K42r are in  
lower case letters.)

A further PCR primer, 3K2, was designed to produce an 82 base-pair amplicon when amplified with 5K42r. The sequence of 3K2 is:

5' CGTCCACAAAATGATTCTGA 3' "3K2" Primer

The primers and DNAzyme were synthesised by Pacific Oligos Pty. Ltd. (Lismore, NSW, Australia) or Oligos Etc., Inc. (Wilsonville, OR, USA). The DNAzyme Dz1 was modified by adding a 3' phosphate group to prevent extension by Taq DNA polymerase. The 5' primer, 5K42r, was 5' end-labelled with gamma-<sup>32</sup>P by incubating 25 ul of 20 uM primer with 2.5 ul of Polynucleotide Kinase (10 x 10<sup>3</sup> U/ml, 3' phosphatase-free, Boehringer Mannheim), 2.5 ul RNasin (40 U/ml Recombinant RNasin®, Ribonuclease Inhibitor, Promega), 5 ul of polynucleotide kinase buffer (Boehringer Mannheim), 10 ul of gamma-<sup>32</sup>P Adenosine 5'-Triphosphate (2.5 uM, Stable Label Gold™, Bresatec) and 5 ul of DEPC water for 30 minutes at 37°C.

(c) Preparation of K-ras Templates

5 pUC 18 plasmid vectors containing K-ras exon 1 sequences, which were either wild-type (GGT) or mutated at codon 12 (CGT or AGT), were used as DNA templates for PCR.

(d) Detection of Point Mutations

10 PCR mixtures contained 0.2 pg/ $\mu$ l plasmid DNA, 10 pmole of gamma- $^{32}$ P-labelled 5K42r, 2 pmole 3K2, 1 mM DTT, 8 mM MgCl<sub>2</sub>, each dNTP (dATP, dCTP, dTTP, dGTP) at 100  $\mu$ M, 0.4 U/ $\mu$ l RNasin®, and 1 x buffer (100 mM NaCl with 50 mM Tris pH 8.3 at 25°C). Duplicate reactions were set up with 0.5  $\mu$ M Dz1, and single reactions without Dz1 were set up as control reactions. Six units of Taq DNA polymerase (5 U/ $\mu$ l AmpliTaq, Perkin-Elmer) were mixed with TaqStart™ antibody (Clontech) to give a final molar ratio of Taq DNA polymerase:TaqStart™ antibody of 1:5. The Taq DNA polymerase:TaqStart™ antibody mixture was incubated for 15 minutes at room temperature prior to addition to the PCR mix. The total reaction volumes were 50  $\mu$ l. The reactions were placed in a GeneAmp PCR 9600 (Perkin-Elmer) and denatured at 94°C for 2 minutes, then subjected to 15 cycles of 60°C for 1 minute, followed by 94°C for 20 seconds. The reaction was further subjected to 25 cycles of 40°C for 1 minute followed by 94°C for 20 seconds.

15 20 25 30 A 2.5  $\mu$ l aliquot of each reaction was mixed with 2.5  $\mu$ l of loading dye (97.5% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.01 M EDTA), incubated at 75°C for 2 minutes and then loaded 35 immediately onto a pre-warmed 16% denaturing (urea)

acrylamide gel. The gels were electrophoresed for approximately one hour. The PCR product and cleavage fragments were visualised by scanning the gel using a Molecular Dynamics Phosphorimager 445 SI.

5

Several bands were visible on the gel (data not shown). The fragments, in order of mobility from the slowest to the fastest (i.e., from the origin to the bottom of the gel) were (a) PCR amplicons (running as a doublet), (b) unincorporated primer, and (c) cleaved PCR amplicons. Small amounts of two fragments, produced by background hydrolysis at the ribonucleotide residues within the 5' primer, were also visible running between the primer and cleaved amplicons and running parallel with the cleaved amplicons. In all reactions, PCR product and unincorporated primer were visible. Reactions containing template DNA that was wild-type at codon 12 (i.e., fully complementary to the DNAzyme) contained cleaved amplicons. Reactions containing template DNA that was mutated at codon 12 (i.e., mismatched with the DNAzyme) did not contain cleaved amplicons. Only low levels of background hydrolysis products were visible at this position on the gel in these reactions.

25

(2) DNAzymes targeting mutations at K-ras codon 12; induced cleavage site

(a) Strategy

30

PCR using a 5' DNA/RNA chimeric primer (5K44r) and a 3' primer (3K2) amplified a region of the K-ras gene. 5K44r hybridized to the K-ras sequence adjacent to codon 12 and contained the purine:pyrimidine residues which formed the potential DNAzyme cleavage site. The purine ribonucleotide in 5K44r was mismatched with respect to the K-ras template where the wild-type sequence has a

pyrimidine at this position. This primer therefore induces a DNAzyme cleavage site. Extension from the 3' end of 5K44r by Taq DNA polymerase amplified codon 12 of the K-ras gene. A DNAzyme, Dz3, was designed to cleave 5 amplicons that harbor wild-type sequence at codon 12 of K-ras. The 5' arm of the DNAzyme was fully complementary to sequences that are wild-type at codon 12. Mutations at K-ras codon 12, which result in mismatches with the 5' DNAzyme-hybridizing arm, were 10 predicted to significantly decrease the efficiency of DNAzyme cleavage.

(b) Design of PCR Primers and DNAzymes

15    5' TATAAACTTGTGGTAGTTGGAGCTGTGGCGTAGGCAA 3'              K-ras  
 5' TATAAACTTGTGGTAGTTGGAGGu 3'                          "5K44r" Primer  
 3'            GAACACCATCAACCTC ACCACCGC 5'              "Dz3" DNAzyme  
 A G  
 G G  
 C C  
 A T  
 A A  
 C G  
 A T C

(Codon 12 in the K-ras wild-type sequence is underlined, and the ribonucleotide bases in the primer 5K44r are in lower case letters. The ribonucleotide "g" in the primer 5K44r is mismatched with respect to the K-ras sequence.)

A further PCR primer, 3K2, was designed to produce an 82 base-pair amplicon when amplified with 5K44r. The sequence of 3K2 is:

35' CGTCCACAAATGATTCTGA 3' "3K2" Primer

The primers and DNAzyme were synthesised by Pacific Oligos Pty. Ltd. (Lismore, NSW, Australia) or Oligos Etc., Inc. (Wilsonville, OR, USA). The DNAzyme Dz3 was

modified by adding a 3' phosphate group to prevent extension by Taq DNA polymerase. The 5' primer, 5K44r, was 5' end-labelled with gamma-<sup>32</sup>P by incubating 25 ul of 20 uM primer with 2.5 ul of polynucleotide kinase (10 x 5  $10^3$  U/ml, 3' phosphatase-free, Boehringer Mannheim), 2.5 ul RNasin (40 U/ml Recombinant RNasin®, Ribonuclease Inhibitor, Promega), 5 ul of polynucleotide kinase buffer (Boehringer Mannheim), 10 ul of gamma-<sup>32</sup>P Adenosine 5'-Triphosphate (2.5 uM, Stable Label Gold™, Bresatec) and 5 ul of DEPC water for 30 minutes at 37°C.

(c) Preparation of K-ras Templates

pUC 18 plasmid vectors containing K-ras exon 1 sequences, which were either wild-type (GGT) or mutated at codon 12 (CGT), were used as DNA templates for PCR.

(d) Detection of Point Mutations

PCR mixtures contained 0.2 pg/ul plasmid DNA, 10 pmole of gamma-<sup>32</sup>P-labelled 5K44r, 2 pmole 3K2, 1 mM DTT, 8 mM MgCl<sub>2</sub>, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 uM, 0.4 U/ul RNasin®, and 1 x buffer (100 mM NaCl with 50 mM Tris pH 8.3 at 25°C). Duplicate reactions were set up with 0.5 uM Dz3 DNAzyme, and single reactions without Dz3 were set up as control reactions. Six units of Taq DNA polymerase (5 U/ml AmpliTaq, Perkin-Elmer) were mixed with TaqStart™ antibody (Clontech) to give a final molar ratio of Taq DNA polymerase:TaqStart™ antibody of 1:5. The Taq DNA polymerase:TaqStart™ antibody mixture was incubated for 15 minutes at room temperature prior to addition to the PCR mix. The total reaction volumes were 50 ul. The reactions were placed in a GeneAmp PCR 9600 (Perkin-Elmer) and denatured at 94°C for 2 minutes, 35 then subjected to 30 cycles of 60°C for 1 minute,

followed by 94°C for 20 seconds. The reaction was further subjected to 10 cycles of 50°C for 1 minute followed by 94°C for 20 seconds.

5        A 2.5 ul aliquot of each reaction was mixed with 2.5 ul of loading dye (97.5% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.01 M EDTA), incubated at 75°C for 2 minutes and then loaded immediately onto a pre-warmed 16% denaturing (urea) 10 acrylamide gel. The gels were electrophoresed for approximately 1 hour. The PCR product and cleavage fragments were visualised by scanning the gel using a Molecular Dynamics Phosphorimager 445 S1.

15       Several bands were visible on the gel (data not shown). The fragments, in order of mobility from the slowest to the fastest (i.e., from the origin to the bottom of the gel) were (a) PCR amplicons (running as a doublet), (b) unincorporated primer, and (c) cleaved PCR 20 amplicons. Small amounts of a fragment, produced by background hydrolysis at the ribonucleotide bond within the 5' primer, was also visible running parallel with the cleaved amplicons. In all reactions, PCR product and unincorporated primer were visible. Reactions containing 25 template DNA that was wild-type at codon 12 (i.e., fully complementary to the DNAzyme) contained cleaved amplicons. Reactions containing template DNA that was mutated at codon 12 (i.e., mismatched with the DNAzyme) did not contain cleaved amplicons. Only low levels of background 30 hydrolysis products were visible at this position on the gel in these reactions.

B. DNAzymes targeting mutations at K-ras codon 12; cleavage in cis orientation

Cleavage of amplicons produced from a chimeric primer can also be achieved using active DNAzymes that are synthesised during PCR. In one example of such a reaction the DNAzyme is cleaving the substrate in the *cis* orientation.

10 (a) Strategy

PCR using a 5' DNA/RNA chimeric primer (5K42r) and a 3' zymogene primer (3K42Dz2) amplified a region of the K-ras gene. 5K42r hybridized to the K-ras sequence adjacent to codon 12 and contained the purine:pyrimidine residues which formed the potential DNAzyme cleavage site. The zymogene primer 3K42Dz2 had a 3' region that was complementary to K-ras, and a 5' region that contained the antisense of a DNAzyme. The zymogene primer had no inherent catalytic activity itself but, when used in conjunction with 5K42r, it facilitated the production of amplicons which had a DNAzyme cleavage site near their 5' termini and active (sense) DNAzymes at their 3' termini. The DNAzyme is designed to cleave the 5' end of the amplicons in *cis*. The 5' arm of the DNAzyme was fully complementary to sequences that are wild-type at codon 12. Mutations at K-ras codon 12, which result in mismatches with the 5' DNAzyme arm, were predicted to significantly decrease the efficiency of DNAzyme cleavage.

(b) Primer Sequences

35 5' chimeric primer 5K42r  
(upper case - deoxyribonucleotide residues; lower case - ribonucleotide residues)

5' TATAAACTTGTGGTAGTTGGAgcT 3'

3' zymogene primer 3K42Dz2  
5 (complement (antisense) of 10:23 catalytic core in bold)

5' ACTTGTGGTAGTTGGATCGTTAGCTAGCCCTGG  
TGGCAGCTGTATCGTCAAGGCACTC 3'

10

The primers were synthesised by Pacific Oligos Pty. Ltd. (Lismore, NSW, Australia) or Oligos Etc., Inc. (Wilsonville, OR, USA). The 5' primer, 5K42r, was 5' end-labelled with gamma-<sup>32</sup>P by incubating 25 ul of 20 uM primer with 2.5 ul of polynucleotide kinase ( $10 \times 10^3$  U/ml, 3' phosphatase-free, Boehringer Mannheim), 2.5 ul RNasin (40 U/ml Recombinant RNasin®, Ribonuclease Inhibitor, Promega), 5 ul of polynucleotide kinase buffer (Boehringer Mannheim), 10 ul of gamma-<sup>32</sup>P Adenosine 5'-Triphosphate (2.5 uM, Stable Label Gold™, Bresatec) and 5 ul of DEPC water for 30 minutes at 37°C.

(c) K-ras DNA Templates

25 pUC 18 plasmid vectors containing K-ras exon 1 sequences, which were either wild-type (GGT) or mutated at codon 12 (CGT or AGT), were used as DNA templates for PCR.

30 (d) Cleavage in cis by DNAzymes synthesised during the PCR

PCR mixtures contained 0.2 pg/ul K-ras plasmid DNA, 10 pmole of gamma-<sup>32</sup>P-labelled 5K42r, 2 pmole 3K42Dz2, 1 mM DTT, 8 mM MgCl<sub>2</sub>, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 uM, 0.4 U/ul RNasin®, and 1 x buffer (100 mM NaCl

with 50 mM Tris pH 8.3 at 25°C). Duplicate reactions were set up for each DNA template. Six units of Taq DNA polymerase (5 U/ul AmpliTaq, Perkin-Elmer) were mixed with TaqStart™ antibody (Clontech) to give a final molar ratio of Taq DNA polymerase:TaqStart™ antibody of 1:5. The Taq DNA polymerase:TaqStart™ antibody mixture was incubated for 15 minutes at room temperature prior to addition to the PCR mix. The total reaction volumes were 50 ul. The reactions were placed in a GeneAmp PCR 9600 (Perkin-Elmer), denatured at 94°C for 2 minutes, subjected to 30 cycles at 60°C for 1 minute, followed by treatment at 94°C for 20 seconds. The reaction was further subjected to 10 cycles at 50°C for 1 minute, followed by treatment at 94°C for 20 seconds.

15

A 2.5 ul aliquot of each reaction was mixed with 2.5 ul of loading dye (97.5% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.01 M EDTA), incubated at 75°C for 2 minutes, and then loaded 20 immediately onto a pre-warmed 16% denaturing (urea) acrylamide gel. The gels were electrophoresed for approximately 1 hour. The PCR product and cleavage fragments were visualized by scanning the gel using a Molecular Dynamics Phosphorimager 445 S1.

25

Several bands were visible on the gel (data not shown). The fragments, in order of mobility from the slowest to the fastest (i.e., from the origin to the bottom of the gel) were (a) PCR amplicons (running as a doublet), (b) unincorporated primer, and (c) cleaved PCR amplicons. Small amounts of two fragments, produced by background hydrolysis at the ribonucleotide residues within the 5' primer, were also visible running between the primer and cleaved amplicons and running parallel 30 with the cleaved amplicons. In all reactions, PCR 35

product and unincorporated primer were visible. Reactions containing template DNA that was wild-type at codon 12 (i.e., fully complementary to the DNAzyme) contained cleaved amplicons. Reactions containing 5 template DNA that was mutated at codon 12 (i.e., mismatched with the DNAzyme) did not contain cleaved amplicons. Only low levels of background hydrolysis products were visible at this position on the gel in these reactions.

10

The sequence below is an amplicon that is wild-type at position 1 of codon 12 (underlined) shown in a conformation wherein the DNAzyme (bold) is hybridizing in *cis*.

15

5' TATAAAACTTGTGGTAGTTGGAgcTGGTGGCGTAGGCAAGAGTGC  
3' TGAACACC**ATCAACCT GACCACCGTCGACATAGCAGTT**  
A G  
20 G G  
C C  
A T  
A A  
C G  
25 A T C

V. Conclusion

30

The instant diagnostic methods are advantageous. Catalytic nucleic acids can require as few as two base pairs of specific sequence to create a cleavage site. Catalytic nucleic acid dinucleotide cleavage sites occur 35 naturally at a greater frequency than do restriction enzyme cleavage sites. Furthermore, mismatched primers can be used to induce cleavage sites for catalytic nucleic acids in the same way that mismatched primers have been used to induce artificial restriction enzyme 40 cleavage sites.

Examples of catalytic nucleic acids which require only a dinucleotide sequence at the cleavage site are the hammerhead ribozyme and the 10-23 DNAzyme. Both 5 these molecules also require complementarity between the hybridizing regions (arms) and the molecule to be cleaved. However these regions can be made target-specific. Although catalytic nucleic acid molecules can only cleave single-stranded nucleic acid templates, 10 methods of generating suitable single-stranded templates are well known in the art. For example, single-stranded RNA templates can be generated by a protocol such as TMA, and single-stranded DNA can be generated by asymmetric PCR (37) or by the denaturation of double- 15 stranded products.

The instant methods provide a new tool for sequence analysis that is potentially more flexible than analysis by RFLP. The combination of nucleic acid amplification 20 with catalytic nucleic acid cleavage overcomes the limitations of analysis using restriction enzymes. Here, the minimum sequence requirement for cleavage has been reduced. Furthermore, since the catalytic nucleic acid must also be complementary in the hybridizing 25 region, these regions which flank the dinucleotide cleavage site will also effect cleavage efficiency. The length of sequence scanned by one catalytic nucleic acid can therefore be greater than that scanned by a single restriction enzyme. The analysis of sequences using 30 catalytic nucleic acids also has an advantage over other protocols since here, no protein enzymes (e.g., restriction enzymes or RNAase A) or toxic compounds are required.

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What is claimed is:

1. A method of determining whether a subject is afflicted with a disorder characterized by the presence of a known nucleic acid mutation, which comprises the steps of
  - (a) isolating a sample of nucleic acid molecules from the subject;
  - (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the mutation in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and
  - (c) determining whether the catalytic nucleic acid molecule in step (b)(ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.
2. A method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of
  - (a) isolating a sample of nucleic acid molecules from the subject;
  - (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii)

under suitable conditions, contacting the resulting amplified segment with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and

(c) determining whether each of the catalytic nucleic acid molecules in step (b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

3. A method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of

(a) isolating a sample of nucleic acid molecules from the subject;

(b) (i) amplifying the nucleic acid segments present in the isolated sample, which segments collectively are known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segments with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in one of the nucleic acid segments having one of the known mutations or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed

either subsequent to or concurrently with step  
(i); and

(c) determining whether each of the catalytic nucleic acid molecules in step (b) (ii) cleaves the amplified segment containing its respective target sequence, so as to determine whether the subject is afflicted with the disorder.

5

4. The method of claim 1, 2 or 3, wherein the subject is a human.

10

5. The method of claim 1, 2 or 3, wherein the disorder is selected from the group consisting of cancer, AIDS and cystic fibrosis.

15

6. The method of claim 5, wherein the disorder is cancer.

20

7. The method of claim 1, 2 or 3, wherein the amplified nucleic acid segment is RNA and the catalytic nucleic acid molecule is selected from the group consisting of DNA and RNA.

25

8. The method of claim 1, 2 or 3, wherein the amplified nucleic acid segment is DNA and the catalytic nucleic acid molecule is RNA or DNA.

30

9. The method of claim 1, 2 or 3, wherein (a) the amplification is performed using a polymerase chain reaction; (b) the catalytic nucleic acid molecule is a 10-23 DNAzyme; and (c) the polymerase chain reaction employs a DNA primer suitable for initiating amplification of the segment, which primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the

35

amplified segment recognized and cleaved by the 10-23 DNAzyme.

10. The method of claim 9, wherein the amplified segment  
5 is recognized and cleaved *in trans* by the DNAzyme.
11. The method of claim 9, wherein (a) the polymerase  
10 chain reaction employs a second DNA primer suitable  
for initiating amplification of the segment, which  
second primer comprises a zymogene encoding a 10-23  
DNAzyme such that, upon amplification, the resulting  
amplified nucleic acid molecule comprises the 10-23  
DNAzyme; and (b) the amplified nucleic acid segment  
is recognized and cleaved *in cis* by the DNAzyme.  
15
12. A kit for use in practicing the method of claim 1,  
2 or 3, which comprises (a) a catalytic nucleic  
acid molecule which specifically recognizes and  
cleaves a target sequence present either (i) in a  
20 nucleic acid segment having a mutation known to be  
characteristic of a disorder or (ii) in the  
corresponding wild-type nucleic acid segment, but not  
both, and (b) a nucleic acid reagent suitable for  
use in amplifying the nucleic acid segment containing  
25 the target sequence.
13. A kit for use in practicing the method of claim 9,  
which comprises (a) a 10-23 DNAzyme which  
specifically recognizes and cleaves a target  
30 sequence present either (i) in a nucleic acid segment  
having a mutation known to be characteristic of a  
disorder or (ii) in the corresponding wild-type  
nucleic acid segment, but not both, and (b) a DNA  
primer suitable for initiating amplification of the  
35 segment under polymerase chain reaction conditions,  
which primer contains at least one purine

ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme.

5 14. A kit for use in practicing the method of claim 11, which comprises

(a) a first DNA primer which comprises a zymogene encoding a 10-23 DNAzyme that specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, which first primer is suitable for initiating amplification of the segment under polymerase chain reaction conditions; and

(b) a second DNA primer suitable for initiating amplification of the segment under polymerase chain reaction conditions, which second primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme, such that, upon amplification, (i) the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme, and (ii) the amplified nucleic acid segment is recognized and cleaved in cis by the DNAzyme.

30

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 99/00848

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : C12Q 1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC C12Q 1/68		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, MEDLINE, CA, BIOT; KEYWORDS: DNA ENZYME#, RNA ENZYME#, RIBOZYME#, DNAYME#, ZYMOGENE#, PCR, AMPLIFICATION, LIGASE CHAIN REACTION, MUTATION#, POLYMORPHISM, ALLELE#.		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Nature Biotechnology Vol. 17 No.1 pages 62-66 issued January 1999. Robertson MP et al, "In vitro selection of an allosteric ribozyme that transduces analytes to amplicons." Whole article	1-14
Y	AU 20718/92 A (AKZO NV) 11 March 1993 Claims 6, 7	1-14
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C		<input checked="" type="checkbox"/> See patent family annex
<ul style="list-style-type: none"> <li>* Special categories of cited documents:</li> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier application or patent but published on or after the international filing date</li> <li>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> </ul> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 12 July 1999	Date of mailing of the international search report 28 JUL 1999	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer <b>ROSS OSBORNE</b> Telephone No.: (02) 6283 2404	

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 99/00848

<b>C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to claim No.</b>
Y	WO 95/19448 A (JOHN HOPKINS SCHOOL OF MEDICINE) 20 July 1995 claims	1-14
P, A	Biochem Biophys Res Commun Vol. 249, No. 3, pages 804-810, issued 28 August 1998 Phylactou LA et al "Hammerhead ribozymes targeted to the FBN1 mRNA can discriminate a single base mismatch between ribozyme and target"	
P,A	WO 99/09162 A (US SECRETARY OF HEALTH & HUMAN SERVICES) 25 February 1999	

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/IB 99/00848

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
AU	20718/92	CA	2075147	EP	525882	FI	922447
		JP	5219999	US	5834255	ZA	9205631
WO	9519448	AU	16038/95	CA	2181147	EP	749495
WO	9909162	AU	90162/98				

END OF ANNEX